

# **Exhibit D**

# Apoptosis in sepsis: a new target for therapeutic exploration

CAROLINE OBERHOLZER,\* ANDREAS OBERHOLZER,\* MICHAEL CLARE-SALZLER,\*,† AND LYLE L. MOLDAWER\*,†

Departments of \*Surgery and †Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida 32610, USA

**ABSTRACT** The treatment of sepsis and septic shock remains a clinical conundrum, and recent prospective trials with biological response modifiers aimed at the inflammatory response have shown only modest clinical benefit. Recently, interest has shifted toward therapies aimed at reversing the accompanying periods of immune suppression. Studies in experimental animals and critically ill patients have demonstrated that increased apoptosis of lymphoid organs and some parenchymal tissues contributes to this immune suppression, anergy, and organ system dysfunction. During sepsis syndromes, lymphocyte apoptosis can be triggered by the absence of IL-2 or by the release of glucocorticoids, granzymes, or the so-called 'death' cytokines: tumor necrosis factor  $\alpha$  or Fas ligand. Apoptosis proceeds via auto-activation of cytosolic and/or mitochondrial caspases, which can be influenced by the pro- and anti-apoptotic members of the Bcl-2 family. In experimental animals, not only can treatment with inhibitors of apoptosis prevent lymphoid cell apoptosis; it may also improve outcome. Although clinical trials with anti-apoptotic agents remain distant due in large part to technical difficulties associated with their administration and tissue targeting, inhibition of lymphocyte apoptosis represents an attractive therapeutic target for the septic patient. — Oberholzer, C., Oberholzer, A., Clare-Salzler, M., Moldawer, L. L. Apoptosis in sepsis: a new target for therapeutic exploration. *FASEB J.* 15, 879–892 (2001)

**Key Words:** *Bcl-2 • caspases • lymphocytes • neutrophils*

SEPSIS AFFECTS MORE THAN 500,000 patients in the U.S. annually, and its incidence continues to increase (1). Despite recent progress in antibiotics and critical care therapy, sepsis is still associated with a high mortality rate. Septic shock and sequential multiple organ failure/dysfunction syndrome (MOF/MODS) correlate with poor outcome (2), and septic shock is the most common cause of death in intensive care units (3). Experimental data have provided a solid rationale for innovative therapies and clinical trials, yet the outcome from this syndrome has not improved significantly in the past 40 years (4).

The immunological cascade resulting in the sepsis response can be initiated by tissue injury, ischemia-

reperfusion injury, gram-positive organisms, and fungi as well as gram-negative organisms and their constituent endotoxin. The sepsis response may begin with an infectious nidus, which may either invade the bloodstream, leading to dissemination and positive blood cultures, or proliferate locally and release various microbial products into the bloodstream. In multiple trauma or hemorrhagic shock, the direct tissue or secondary ischemia-reperfusion injury may also lead to an increased appearance of microorganisms and exotoxins from the gut. The host response to these microbial products or to the trauma and ischemia-reperfusion injury itself leads to the rapid activation of the innate immune response and the release of a variety of humoral mediators, including glucocorticoids, catecholamines, and proximal proinflammatory cytokines like tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1), and IL-6 (5).

A vigorous induction of the innate immune system can and often does have catastrophic effects on the patient with sepsis syndrome. Exaggerated production of proinflammatory cytokines and the induction of more distal mediators such as nitric oxide, platelet activation factor, and prostaglandins have been implicated in the endothelial changes and induction of a procoagulant state that leads to hypotension, inadequate organ perfusion, and necrotic cell death associated with MODS. This proinflammatory state has been defined as being a systemic inflammatory response syndrome (SIRS) (6).

However, most patients survive this initial SIRS event and the proinflammatory state ultimately resolves. The proinflammatory cytokines and humoral mediators responsible for the induction of the innate immune response and SIRS also contribute to the development of acquired or specific immune defects. The patient frequently enters an immunological state characterized by T cell hyporesponsiveness, anergy, and a defect in antigen presentation that has been recently termed a compensatory anti-inflammatory response syndrome (CARS) (7).

<sup>†</sup> Correspondence: Department of Surgery, University of Florida College of Medicine, Room 6116, Shands Hospital, 1600 S.W. Archer Rd., PO Box 100286, Gainesville, FL 32610-0286, USA. E-mail: moldawer@surgery.ufl.edu

The role that apoptosis plays in sepsis syndromes and in the development of CARS and MODS has not been adequately explored, but there is rapidly developing evidence to suggest that increased apoptotic processes may play a determining role in the outcome to sepsis syndromes. In particular, increased apoptosis, particularly in lymphoid tissues and potentially in some parenchymal tissues from solid organs, may contribute to the sepsis-associated MODS and can be a potential therapeutic target for intervention. Direct apoptotic organ injury and the immune suppression secondary to apoptotic losses in T cell, B cell, and NK cell populations may contribute significantly to the risk of secondary opportunistic infections. Therapeutic efforts at modulating the apoptotic response, particularly by interfering with cell signaling pathways that lead to caspase-mediated apoptosis, represent an attractive therapeutic target for the septic patient. In the current review, we address the scientific rationale for modifying apoptotic pathways in sepsis syndrome and propose several potential therapeutic targets.

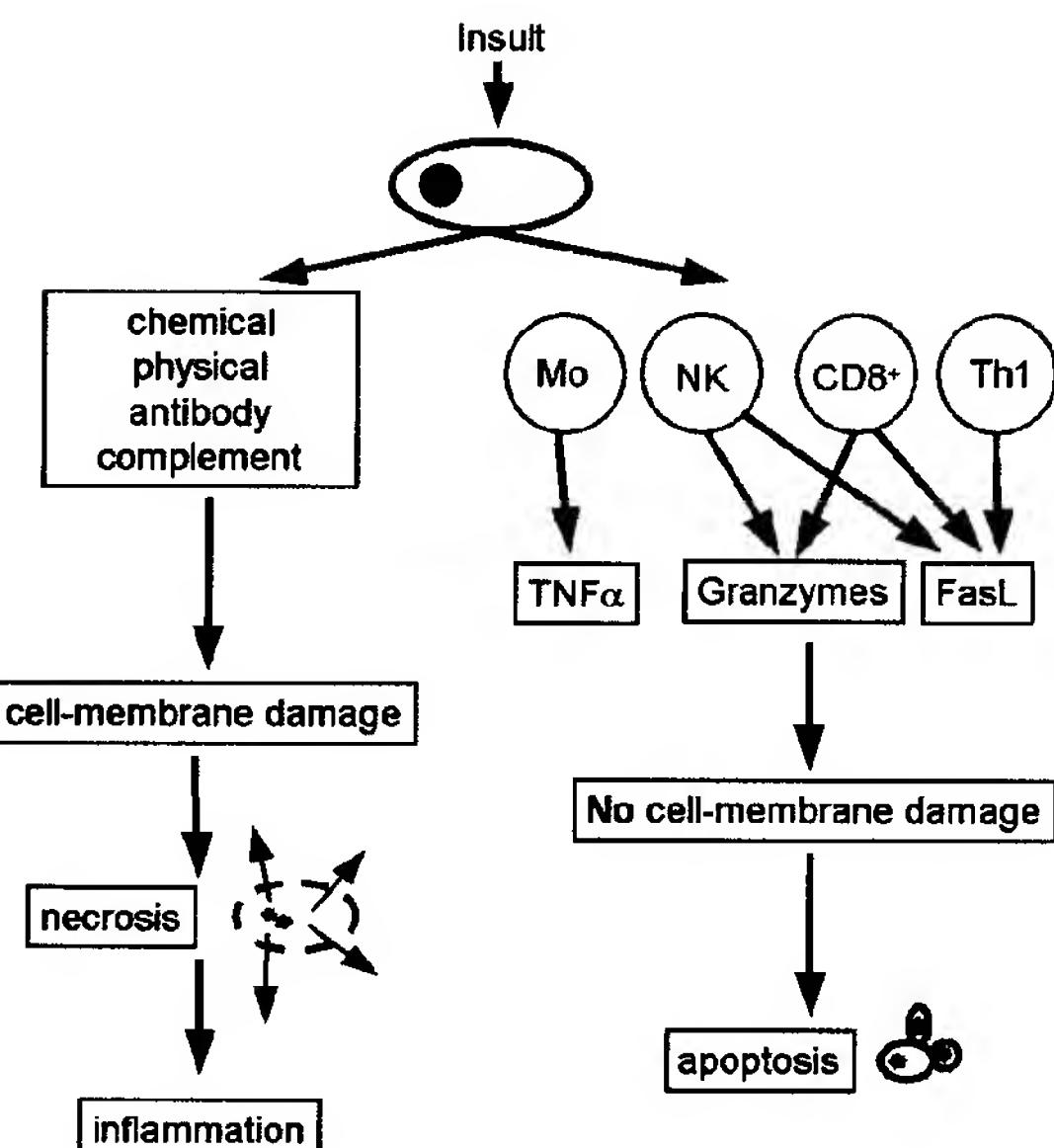
## APOPTOSIS DURING INFLAMMATION

Cells can die in two ways: by damaging their cell membrane and then undergoing necrosis or by shrinking and blebbing the intact cell membrane, which leads to apoptosis (Fig. 1). Physical or chemical injury, such as the deprivation of oxygen that occurs in the heart muscle during myocardial infarction or in sepsis when the microvasculature is disrupted by fibrin deposition and intravascular coagulopathies, often leads to cell disintegration or necrosis. The necrotic tissue is phagocytosed and degraded by phagocytic cells, often leading to inflammation.

An alternative form of cell death is known as programmed cell death or apoptosis. Apoptosis is a normal cellular process that is crucial for tissue remodeling and/or development. For example, most thymocytes undergo an apoptotic cell death when they fail positive selection or are negatively selected as a result of recognizing self-antigens (8).

During apoptosis, cells undergo a non-necrotic cellular suicide that, in contrast to necrosis, generally does not produce inflammation and injury in the tissue (9). Cells undergoing apoptosis typically show DNA fragmentation, condensation of chromatin, membrane blebbing, cell shrinkage, and finally, disassembly into membrane-enclosed vesicles (10). A hallmark of this type of cell death is the fragmentation of nuclear DNA into multiples of 200 base pairs through the activation of endogenous nucleases that cleave the DNA between nucleosomes.

Cytokines like TNF- $\alpha$  and Fas ligand (FasL), glucocorticoids and granzymes can induce apoptosis in several cell populations, whereas other cytokines such as IL-1, IL-6, and G-CSF often inhibit apoptosis. Increased levels of several of these proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, G-CSF) have been reported



**Figure 1.** Insult-dependent pathways leading to necrosis or apoptosis. Cells can die in two ways: either by cell membrane damaging mechanisms like chemical, physical, complement, or antibody injury, leading to cellular necrosis, or by programmed cell death (apoptosis) induced by soluble or membrane-bound tumor necrosis factor (TNF)  $\alpha$  or Fas ligand (FasL) produced by macrophages (Mo), cytotoxic-T cells (CD8 $+$ ), or T helper cells (Th1). Moreover, cytotoxic T cells (CD8 $+$ ) or natural killer (NK) cells can induce apoptosis in the target cells through the release of granzymes.

in sepsis syndromes and have been implicated as principal effectors of the host immune responses during endotoxemia (11) and severe trauma (12). In addition, increased FasL expression has been reported in rodent models of endotoxemia and in generalized peritonitis (13); elevated soluble Fas ligand has also been detected in critically ill patients [head injury and acute respiratory distress syndrome (ARDS)] in cerebrospinal fluid as well as in bronchial fluid (14, 15).

## TUMOR NECROSIS FACTOR-INDUCED APOPTOSIS AND SIGNAL TRANSDUCTION

There is growing appreciation that alterations in lymphoid cell and neutrophil apoptosis are normal components of the innate immune response. As such, it is not surprising that several of the humoral factors that initiate and regulate the innate immune response also regulate apoptotic processes in lymphoid tissues and in neutrophils. TNF- $\alpha$ , FasL, and glucocorticoids appear to be the principal humoral factors that may induce apoptosis in these cell populations.

TNF- $\alpha$  is produced by macrophages and T cells in response to infection (16). TNF- $\alpha$  is involved in both inflammation and cellular apoptosis, and is synthesized as a 26 kDa membrane-associated precursor that is cleaved to the soluble 17 kDa form by TNF- $\alpha$ -converting enzyme, a novel matrix metalloproteinase (17). The biological responses to TNF- $\alpha$  are mediated by ligand

binding via two structurally distinct receptors: p55 (TNF-R I, CD120a) and p75 (TNF-R II, CD120b) (18). Expression of these two receptors appears to be differentially regulated and shows some tissue specificity. Both receptors are transmembrane glycoproteins (Fig. 2). The two TNF receptors, however, differ significantly in their binding affinities (19) as well as their intracellular signaling pathways (20). The intracellular signaling domains of the TNF-R1 (p55) receptor actually shares greater homology with the intracellular signaling domains of Fas/CD95 receptor than it does with TNF-R2 (p75), particularly with regard to a highly conserved intracellular domain called the 'death domain' (DD). This sequence plays a pivotal role in TNF- $\alpha$ 's ability to trigger apoptosis in the cell.

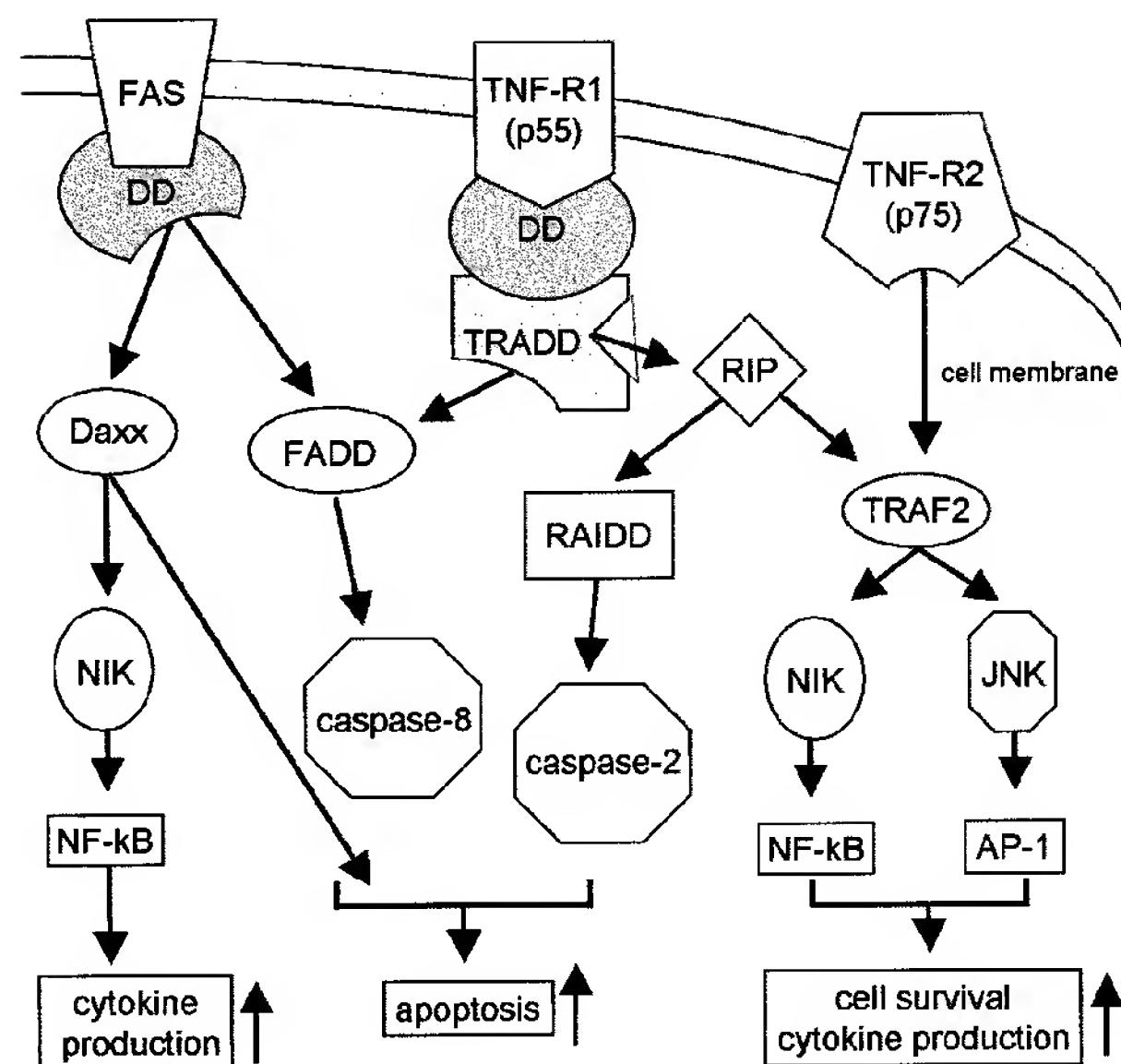
Binding of homotrimeric TNF- $\alpha$  causes trimerization of its receptors. Trimerization of the TNF-R1 (p55) (18) initiates the physical association of the receptors' death domains. Subsequently, an adapter protein termed TRADD (TNF receptor 1-associated death domain) binds through its own death domain to the

clustered receptor death domains of the TNF-R1. TRADD signaling lies at the bifurcation of the apoptotic and proinflammatory transduction pathways of TNF- $\alpha$  (Fig. 2) (21). TRADD functions as a platform adapter that recruits several signaling molecules to the activated receptor like TRAF2 (TNF receptor-associated factor 2) (22) and RIP (receptor interacting protein) (23) stimulating the pathway, leading to either nuclear factor (NF)- $\kappa$ B-inducing kinase (NIK) to activate NF- $\kappa$ B or c-Jun N-terminal kinase to activate activator protein 1 (AP-1) (24). On the other hand, binding of Fas-associated death domain (FADD) mediates activation of apoptosis (25). FADD couples the TNF-R1-TRADD complex to induce the recruitment and activation of procaspase-8 molecules (26). Activation of caspase-8, also known as FADD-like IL- $\beta$ -converting enzyme by the FADD complex is a principal mechanism initiating physiological and pathophysiological apoptosis through caspase-3 (Fig. 2) (27). Moreover, FADD represents the convergence point for signaling between the p55 TNF receptor and Fas receptor (27). Besides FADD, TNF-R1 can engage an adapter called RIP-associated ICH-1/ZED-3-homologous protein with a death domain (RAIDD) or caspase and RIP adapter with death domain (28). RAIDD binds through a death domain to the death domain of RIP and through a caspase recruitment domain (CARD) motif to a similar sequence in the death effector, caspase-2, thereby inducing apoptosis. The TNF type II receptor participates in the proinflammatory signal of TNF- $\alpha$  via TRAF2, which mediates activation of NF- $\kappa$ B (29).

#### FAS LIGAND-INDUCED APOPTOSIS AND SIGNAL TRANSDUCTION

Fas ligand (FasL) is a 40 kDa type II integral membrane protein belonging to the TNF/nerve growth family (30). Membrane-bound FasL can be proteolytically cleaved by metalloproteinases generating a soluble FasL (31). The specific receptor for FasL is Fas (CD95, Apo-1), a 45 kDa type I transmembrane protein and a member of the TNF receptor family (32). Fas is ubiquitously expressed in various tissues with an abundant expression in the thymus, liver, heart, and kidney and is controlled mainly by tight regulation of FasL expression (33). On the other hand, FasL is predominantly expressed in activated lymphocytes and natural killer (NK) cells and constitutively expressed in tissues such as testis and eye. The Fas and FasL pathway plays an important role in physiological processes that require apoptosis. These processes include the peripheral deletion of activated mature T cells at the end of an immune response, or the killing of virus-infected or cancer cells by cytotoxic T cells and by natural killer cells, and the killing of inflammatory cells at 'immune privileged' sites (34).

Fas ligand binding to Fas leads to oligomerization and clustering of the Fas DD (Fig. 2). The DD are actually a protein-protein interaction domain that binds an adapter protein FADD (35). The resulting



**Figure 2.** Intracellular signaling of the two death cytokines: FasL and TNF- $\alpha$ . FasL binds to its receptor Fas (CD95), which induces apoptosis in the target cell through binding of FADD (Fas-associated death protein) to its receptors death domain (DD) and therefore activates caspase-8. Moreover, Daxx can bind to CD95, which activates c-Jun N-terminal kinase (JNK) and caspases. Conversely, TNF- $\alpha$  can either bind to TNF receptor 1 (TNF-R1, p55) or TNF receptor 2 (TNF-R2). The activation of TNF-R1 leads to either to cell apoptosis through activation of caspase-8 or caspase-2 or to the production of proinflammatory cytokines through activation of either NIK (and therefore activation of NF- $\kappa$ B) or through activation of activator protein-1 (AP-1). The pathways are dependent on the intracellular signaling transduction protein binding to TNF receptor-associated death domain (TRADD). Intracellular signaling transduction proteins include FADD (Fas-associated death protein), RIP (receptor-interacting protein), RAIDD (RIP-associated ICH-1/CED-3-homologous protein with death domain), and TRAF2 (TNF receptor-associated factor).

complex is termed the death-inducing signaling complex. FADD also contains a death effector domain that acts as a CARD-activating procaspase-8 (36). Caspase-8 then activates downstream effector caspases. The net results of these enzymes is induction of apoptosis, which is associated with the exposure of phosphatidyl serines on the exterior cell surface promoting rapid phagocytosis of dying cells. Ligation of FasL to its receptor leads to apoptosis by at least two different pathways depending on the cell type (37). T cell lines that overexpress Bcl-2 are not susceptible to CD95-induced apoptosis (38). In contrast, Bcl-2 overexpression has no influence on Fas-mediated apoptosis in the SKW6 cell line (37). In the former, it is believed that ligation of FasL to the Fas receptor leads to mitochondrial release of cytochrome *c* and the cleavage of caspase-3. In the latter cell line, CD95 leads to caspase-8 activation, which then bypasses the mitochondria and directly results in activation of the downstream mediators, such as caspase-3 (37) (Fig. 2).

Giordano et al. have shown that Fas expression can be induced and up-regulated by inflammatory cytokines such as IL-1 (39), which are elevated during sepsis and trauma (11, 40). This raises the intriguing possibility that inflammation per se may promote Fas-dependent injury. In addition, soluble FasL has been detected in cerebrospinal fluid in patients after severe head injury (14) and soluble FasL concentrations correlated with the severity of brain injury. Furthermore, increased FasL has been detected in bronchial fluid from patients suffering ARDS (15).

Moreover, Ottonello and colleagues have shown that soluble FasL is a potent chemoattractant for human neutrophils without evoking their secretory responses (41) and thus further supports the Fas-FasL system-mediated cell injury independent of its apoptosis-inducing properties.

#### CYTOTOXIC T CELLS (CD8) CAN INDUCE TARGET CELLS TO UNDERGO PROGRAMMED CELL DEATH IN SEPSIS SYNDROMES

Cytotoxic T lymphocytes and NK cells are known to induce lethal damage on their target cells by inducing granule exocytosis of granzymes or via the FasL system. However, the role that cytotoxic T cells play in the host response to sepsis is unclear. Faist and colleagues have reported that after a burn injury, peripheral blood CD8+ numbers did not change, but their secretion of IL-4 was markedly increased (42). There is strong evidence that cytotoxic T cells kill their targets largely by programming them to undergo apoptosis. When cytotoxic T cells are mixed with target cells and rapidly brought into contact by centrifugation, they can program antigen-specific target cells to die within 5 min, although death may take hours to become fully evident. An early feature of T cell killing is degradation of target cell DNA, while later effects include the loss of membrane integrity, which may also be induced by other cytotoxic mechanisms. The short period required by

cytotoxic T cells to program their targets to die reflects the release of preformed effector molecules by the T cell, which activate an endogenous apoptotic pathway within the target cell. The elimination of infected cells without destruction of healthy tissue requires the cytotoxic mechanisms of CD8+ T cells.

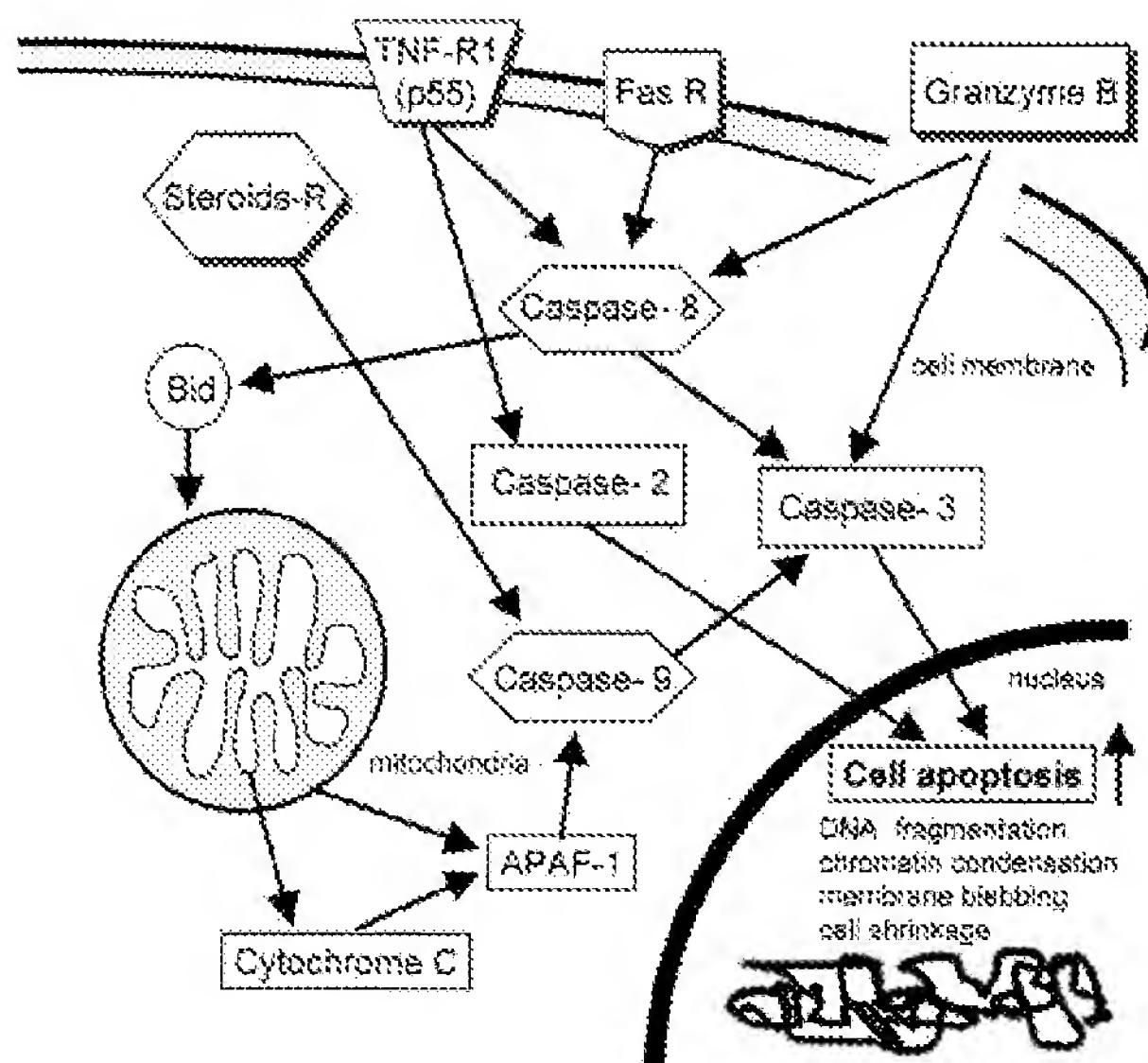
The principal mechanism through which cytotoxic T cells act is by the calcium-dependent release of specialized lytic granules upon recognition of antigen on the surface of a target cell. These granules are modified lysosomes that contain at least two distinct classes of cytotoxins, proteins that are expressed selectively in cytotoxic T cells and stored in the lytic granules in active form. CD8+ T cells carry out their killing function by releasing two types of preformed cytotoxins: granzymes, which induce apoptosis in any target cell, and the pore-forming protein perforin, which creates holes in the target cell membrane through which granzymes can enter. Recently, it has been shown that granzyme B can cleave the ubiquitous cellular enzymes, caspase-3 and caspase-8, which play a key role in the programmed cell death of all cells (43, 44). Ligation of the T cell receptor similarly induces *de novo* synthesis of perforin and granzymes in CD8+ T cells, so that their supply of lytic granules is replenished. This makes it possible for a single CD8+ T cell to kill many targets in succession.

The membrane-bound molecule, FasL, expressed by CD8+ and T<sub>H1</sub> CD4+ T cells is also capable of inducing apoptosis by binding to Fas on target cells. Distribution of Fas on the surface of target cells is generally ubiquitous, so the rate-limiting step is often the up-regulation of Fas ligand on the surface of the effector cells or the recruitment of Fas ligand expressing cells to the tissues. These properties allow the cytotoxic T cell to attack and destroy virtually any cell that is infected with a cytosolic pathogen.

#### GLUCOCORTICOIDS INDUCE APOPTOSIS THROUGH CYTOSOLIC CASPASES

Glucocorticoid hormones have been documented to be increased in sepsis syndromes and have been implicated as regulators of T cell growth, differentiation, and death. In general, T lymphocytes, particularly thymocytes, are especially sensitive to glucocorticoid-mediated apoptosis. The intracellular signaling pathways involved in glucocorticoid-induced apoptosis appear to be distinct from the proximal pathways invoked by either TNF- $\alpha$  or FasL, although both appear to be caspase-3 dependent. Thymocyte apoptosis induced by glucocorticoids can be prevented by the broad-acting caspase inhibitor ZVAD-fmk as well as by a more specific caspase-3 inhibitor, DEVD-CHO.

Unlike apoptosis-inducing pathways invoked by TNF- $\alpha$  or FasL, which require caspase-8, glucocorticoid-mediated activation of caspase-3 appears to be dependent on activation of caspase-9 (Fig. 3). Evidence that caspase-9-mediated activation of caspase-3 is independent of caspase-8 comes from studies of embryonic



**Figure 3.** Receptor-dependent caspase activation in the apoptosis signaling pathway. Cell apoptosis can be induced either through caspase-2 or caspase-3 activation. Caspase-2 is activated through tumor necrosis factor receptor 1 (TNF-R1), whereas caspase-3 is activated either by granzyme B or indirectly by steroids, TNF-R1, Fas receptor (Fas R), or the mitochondrial pathway through activation of caspase-9.

fibroblasts from caspase-9 knockout mice. These cells are resistant to glucocorticoid-induced apoptosis, but are surprisingly sensitive to apoptosis induced by either UV radiation or FasL (45).

There is controversy, however, regarding how caspase-9 is activated in glucocorticoid-induced apoptosis. Resistance to apoptosis in caspase-9 null cells is associated with a retention in mitochondrial membrane potential, despite translocation of cytochrome *c* to the cytosol. These findings suggest that in glucocorticoid-induced apoptosis, caspase-9 activation of caspase-3 occurs downstream of cytochrome *c* (46).

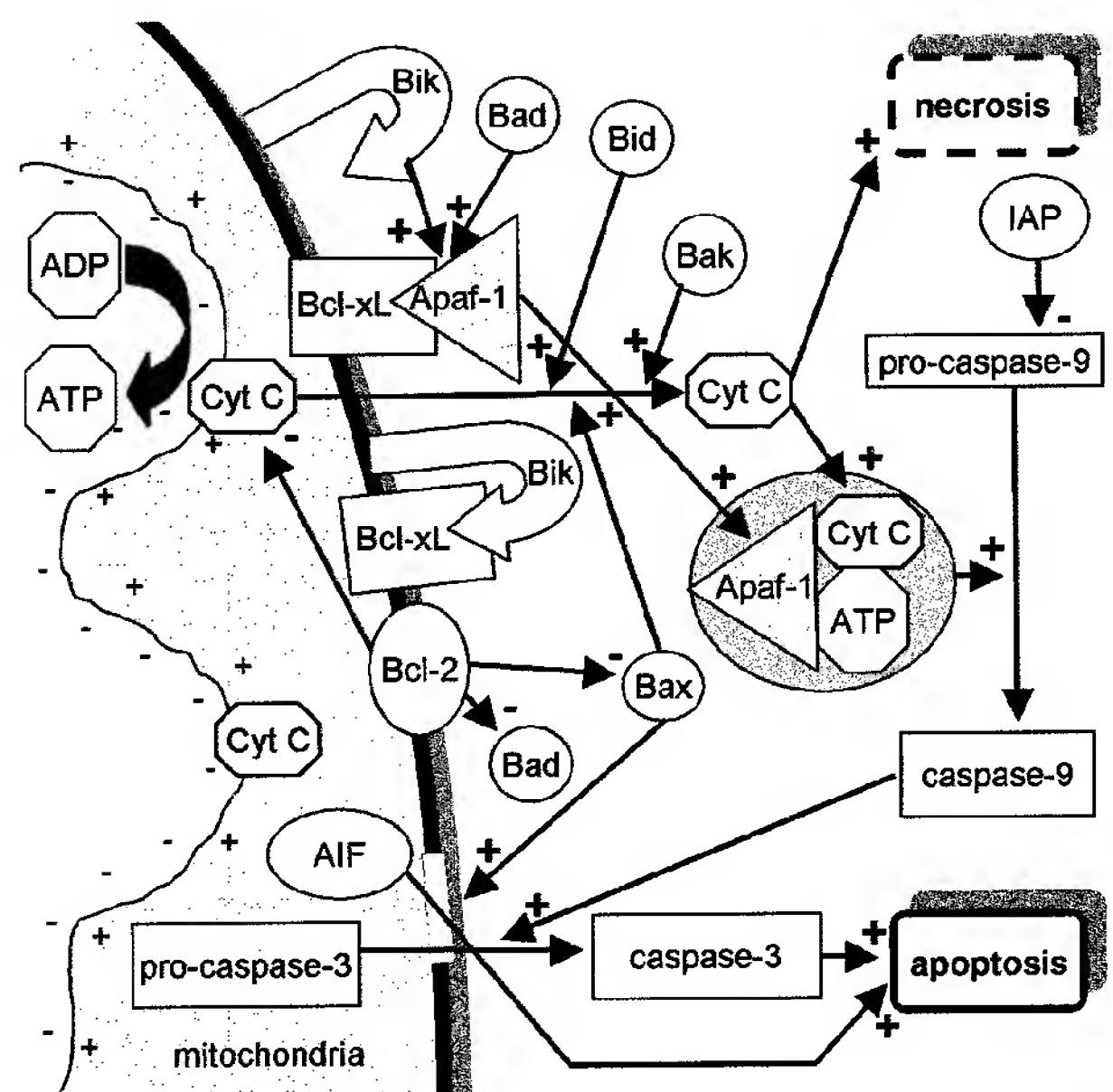
The evidence to date suggests that proximal activation of caspase-9 occurs through acid-sphingomyelinase- and ceramide-dependent pathways. Glucocorticoid binding to its receptor rapidly induces diacylglycerol generation through the actions of a protein kinase C and phospholipase C-dependent event. Diacylglycerol is the primary activator of acid sphingomyelinase and ceramide generation, which appears to be dependent for activation of caspase-9 (47, 48).

### THE ROLE OF CASPASES IN APOPTOSIS

Caspases are constitutively expressed as proenzymes that contain three domains. Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer. Caspases inactivate proteins that protect living cells from apoptosis, and they contribute to cell death by direct disassembly of cell structures (49). It is

not known which caspase is responsible for cleavage of the diverse proteins such as PARP (poly (ADP-ribose) polymerase), lamin B, actin, and others under physiological conditions. Some caspases show overlapping specificities for some substrates (caspase-3 and -7 both cleave PARP), whereas caspase-6 is the only caspase known to cleave lamins (50). Caspases themselves are substrates for other caspases and activate each other in positive feedback loops. Another example of the function of caspases is the cleavage of Bcl-2 proteins, negative regulators of apoptosis, whereby the protein is not only inactivated, but a fragment is also released that may directly promote apoptosis (51). Moreover, caspase activity itself is tightly regulated. By phosphorylation of procaspase-9 through the kinases Akt and p21-Ras, procaspase-9 proteolytic processing through cytochrome *c* is defective (52).

Depending on the signal, different caspases are activated, leading to apoptosis. When ligand binding occurs (FasL, TNF- $\alpha$ ) to their death receptors, caspase-8 is activated; on the other hand, caspase-9 is involved in apoptosis induced by cytotoxic agents (dexamethasone, gamma irradiation) (34, 45). Yet binding of Apaf-1 (apoptosis protease-activating factor 1), cytochrome *c* and dATP must be present for activation of procaspase 9, (Fig. 4) (53). Caspase-9 appears to be an essential requirement for thymocyte apoptosis in stressful conditions (45). Another pathway inducing apoptosis has recently been shown to



**Figure 4.** Influence of the Bcl-2 family proteins in the mitochondrial pathway of apoptosis. The members of the Bcl-2 family exhibit pro- and anti-apoptotic influence by dimerization with one another. Depending on the stimulus (Bid, Bik, Bax, Bad, Bak), cytochrome *c* (Cyt C), apoptosis-inducing factor (AIF), and pro-caspase-3 are released from the mitochondria into the cytoplasm of the cell. This leads to activation of pro-caspase-9 and caspase-3, respectively, inducing cell apoptosis. Depending on the amount of cytochrome *c* available and the quantity of caspase inhibitors, the cell either undergoes necrosis or dies of apoptosis.

be mediated through caspase-12, which is located in the endoplasmic reticulum (ER) and leads to apoptosis by ER stress (i.e., accumulation of excess proteins in the ER). This pathway is independent of the other known caspase-dependent pathways (54).

Some caspases are active before they are proteolytically processed, but do not induce apoptosis because of the presence of endogenous caspase inhibitors, such as members of the inhibitor of apoptosis protein (IAP) family (Fig. 4). These proteins rapidly inactivate active caspases. When a sufficient concentration of activated caspases accumulates and the IAPs cannot neutralize them, apoptosis proceeds (55). In the different pathways inducing apoptosis, caspase-3 appears to play a central role as most pathways result in the activation of caspase-3. The precursor form of caspase-3 is localized in both the cytosol and the intermembrane space of the mitochondria (56).

### THE ROLE OF THE Bcl-2 FAMILY IN APOPTOSIS

Analyses of the proteins that induce or block apoptosis have been extensively explored in the worm, *Caenorhabditis elegans*. In this simpler organism, cellular death is a precisely defined sequence during development, and the consequences of genetic manipulations upon death or survival of these cells has been accurately defined. Several 'ced' genes (*C. elegans* death) have been identified. Remarkably, homologues of these genes have been found in mammalian cells and often serve similar functions. One example of an important effector of apoptosis in the worm is a protein called Ced-3. Its mammalian homologue is an aspartate-directed cysteine protease that belongs to a family of proteases, one prototype of which is an enzyme, called IL-1-converting enzyme (ICE, caspase-1), that converts the precursor form of the cytokines IL-1 $\beta$  and IL-18 to their active forms. Different Ced 3/ICE-like enzymes have been identified. They are normally present in the cytoplasm in an inactive form and are catalytically activated on various stimuli. The targets of these proteases are matrix proteins and nucleoproteins whose degradation results in nuclear DNA fragmentation and apoptosis (57).

Activation of these pro-apoptotic proteases is blocked by a family of proteins, the prototype of which is Bcl-2 (B cell lymphoma-2). Bcl-2 is widely expressed in immature tissues prenatally, but becomes highly restricted with maturation. In the adult, Bcl-2 expression is in immature cell populations, in hormonally responsive epithelia that undergo cycles of hyperplasia and involution, and in neurons of the peripheral nervous system (58). Changes in Bcl-2 expression match cell differentiation more closely than patterns of death. Another member of the Bcl family is Bcl-x, which is present in a long (Bcl-x<sub>L</sub>) and a short form (Bcl-x<sub>S</sub>). The former functions as an anti-apoptotic, like Bcl-2, whereas the latter, as well as a related protein called Bax, promotes cell death. The Bcl-2 family of proteins regulate apoptosis especially in lymphocytes (Fig. 4). Fluctuations in the levels of expression of Bcl-2 or Bcl-x<sub>L</sub> during

lymphocyte maturation and activation appear to correlate inversely with the cell's susceptibility to apoptosis. Forced overexpression of Bcl-2 or Bcl-x<sub>L</sub> results in enhanced survival of immature lymphocytes and prolonged antibody responses (59). On the other hand, disruption of Bcl-2 or Bcl-x<sub>L</sub> leads to reduced survival of mature or immature lymphocytes (60). The family members of Bcl-2 can dimerize with one another, antagonizing or enhancing the function of each other. In this manner, the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo apoptosis (61). Another mechanism to regulate dimerization of Bcl-2 family members is by phosphorylation. Bad, a pro-apoptotic member of the Bcl-2 family, is phosphorylated and loses its ability to bind Bcl-x<sub>L</sub>. This enables the dissociated Bcl-x<sub>L</sub> to execute its anti-apoptotic function.

One of the factors inducing phosphorylation of Bad is IL-3 (62). Further pro-apoptotic members such as Bak and Bax, trigger the release of caspases from death antagonists via heterodimerization and by inducing the release of mitochondrial apoptogenic factors [apoptosis-inducing factors (AIF) and procaspase-3] into the cytoplasm (Fig. 4) (63). In unstimulated cells, Bax is located in the cytosol and is in peripheral association with intracellular membranes, including mitochondria, but inserts into mitochondrial membranes after initiation of a death signal (64). Bax can heterodimerize with multiple anti-apoptotic members and also induce apoptosis by directly inducing cytochrome *c* release (65). On the other hand, heterodimerization with Bcl-2 leads to inhibition of its apoptotic function (66). Bcl-2 and Bcl-x<sub>L</sub> are localized to the outer mitochondrial membranes and endoplasmic reticulum as well as nuclear membranes. The Bcl-2 family members may also act as ion channels, which may play a role in the cell death pathway (67). Bcl-x<sub>L</sub> binds to one portion of Apaf-1, whereas procaspase-9 binds to its NH<sub>2</sub>-terminal CARD. Bcl-x<sub>L</sub> may inhibit the association of Apaf-1 with procaspase-9 and thereby prevent its activation (68). Conversely, Bik, a pro-apoptotic protein, may free Apaf-1 from the death inhibitor (69). When Apaf-1 is freed from Bcl-x<sub>L</sub>, it forms a complex with cytochrome *c* and dATP and activates procaspase-9, which then leads to activation of caspase-3 initiating apoptosis (Figs. 3 and 4).

BAR (bifunctional apoptosis regulator) is an apoptosis modulator cross-linking two apoptosis pathways, namely, the cytosolic and the mitochondrial pathway. It contains a DED-like domain capable of suppressing apoptosis signaling through Fas (cytosolic), as well as a domain that mediates interactions with the Bcl-2 family members and suppresses Bax-induced apoptosis in yeast and mammalian cells (mitochondrial) (70).

### THE ROLE OF MITOCHONDRIA IN APOPTOSIS

At least three general mechanisms are known by which the mitochondria are involved in activation-induced cell death, including 1) the release of proteins that

trigger activation of the caspase family of proteases, 2) disruption of electron transport, oxidative phosphorylation and adenosine triphosphate (ATP) production, and 3) alteration of cellular reduction-oxidation (redox) potential (71). During apoptosis, Bid, a member of the pro-apoptotic Bcl-2 family, is activated as a result of caspase-8 cleavage (Fig. 3). This leads to cytochrome *c* release (72), which can be inhibited by the presence of Bcl-2 on these organelles (73). Once cytochrome *c* is released, the cell either dies by a rapid apoptotic mechanism involving Apaf-1-mediated caspase activation or through a slower necrotic process. The latter is a result of a collapse of electron transport that occurs when cytochrome *c* is depleted from the mitochondria, resulting in a variety of sequelae including generation of oxygen free radicals and decreased production of ATP (Fig. 4). The consequence of this release may depend on the cell type. If endogenous caspase inhibitors (IAP) are present in sufficient quantities, the release of cytochrome *c* may fail to induce the caspase-dependent apoptosis and the slow loss of the electron transport chain may lead to cellular necrosis. Conversely, if cytochrome *c* is available in excess, caspases can be activated and still enough cytochrome *c* may be docked by its high-affinity binding sites to maintain electron transport. ATP production can therefore be continued while caspases are activated and induce apoptosis through cleavage of cytosolic and nuclear substrates (71).

Cytochrome *c*, Apaf-1, and dATP form a complex that activates caspase-9 (Figs. 3 and 4) (74, 75). Apaf-1 has been shown to also interact with other caspases (caspases 4 and 8), upstream of caspase 9 (75). The relevance of these interactions is unknown. Another caspase-activating protein released from mitochondria is AIF. *In vitro*, AIF has been shown to process purified procaspase-3. Its activity is blocked by ZVAD-fmk, a broad caspase inhibitor, raising the possibility that AIF is in reality another caspase (76).

#### LYMPHOCYTE APOPTOSIS IS EXAGGERATED IN THE CRITICALLY ILL

Apoptosis of mature T lymphocytes occurs through at least two distinct processes: antigen-driven and lymphokine withdrawal (Fig. 4). Active T cell apoptosis takes place indirectly either by increased glucocorticoid release or by the antigen-induced expression of death cytokines, like FasL and TNF- $\alpha$  (77, 78). These death cytokines engage specific receptors that assemble caspase-activating protein complexes. Fas-deficient T cells exhibit reduced but clearly evident T cell receptor (TCR)-induced death, and the residual apoptosis is blocked by inhibiting TNF- $\alpha$  (78). In resting T cells, the genes for FasL and TNF are weakly induced by TCR stimulation, but in IL-2 stimulated T cells these death cytokines are induced more strongly (79). This difference can explain in part why TCR engagement kills cycling but not resting T cells, and thereby might explain the observed lymphopenia in septic patients.

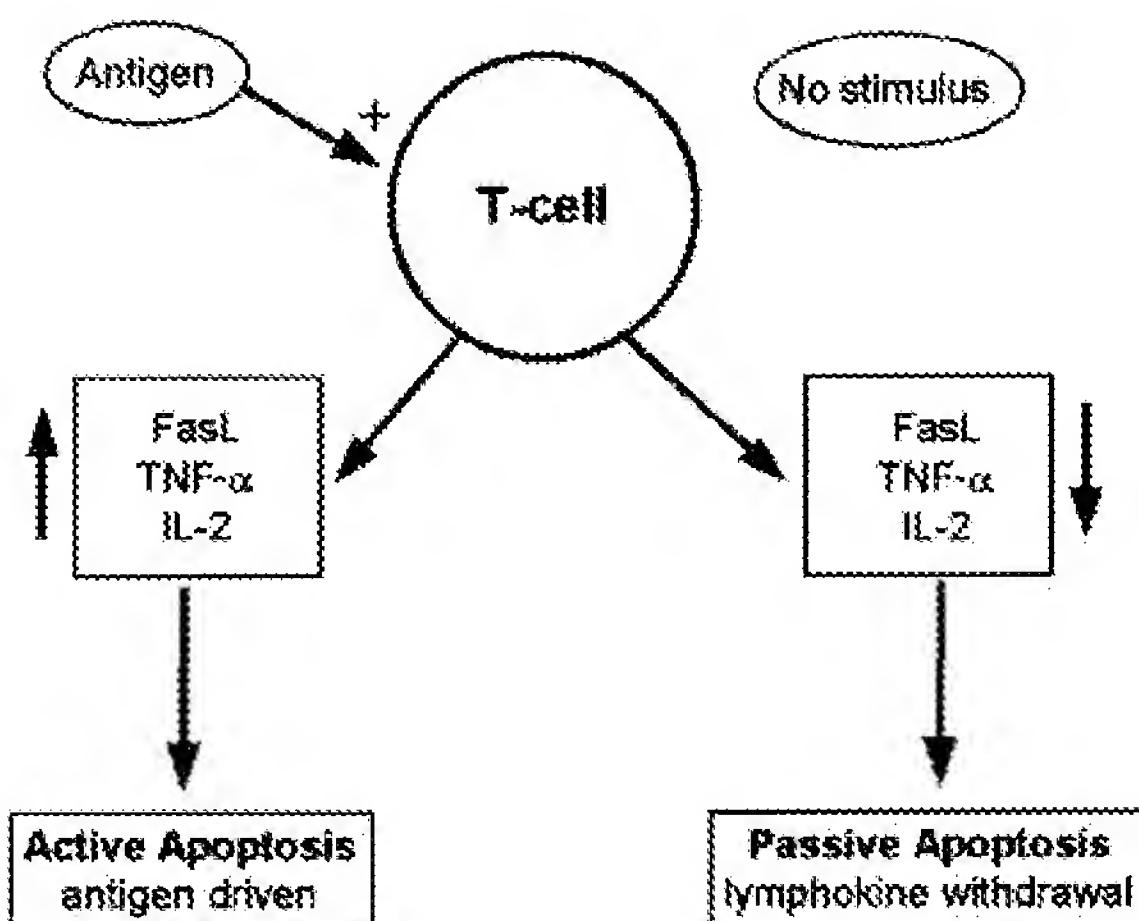
Both FasL and TNF- $\alpha$  are found in cell surface-anchored forms in T cells and can be readily cleaved from the membrane by metalloproteinases. Evidence supports the concept that a single T cell can kill itself through these autocrine signaling pathways (31, 80). However, Fas is constitutively present on circulating T cells and increases during aging (81).

The activation of mature T lymphocytes results in the coexpression of Fas and FasL on the cells. A high concentration of the growth factor IL-2 enhances the expression of FasL on antigen-stimulated T cells and the development of sensitivity to Fas-mediated apoptosis. Thus, IL-2 is both a growth factor for T cells and a feedback regulator of T cell responses. Reactivation, especially in the presence of IL-2, leads to engagement of Fas by FasL and triggering of an apoptotic pathway, which can be responsible for activation-induced cell death and the prevention of uncontrolled activation of lymphocytes. In some cell populations, such as CD8+ T lymphocytes, activation-induced cell death is apparently triggered not through Fas, but through TNF receptor signaling. Conversely, apoptosis of CD4+ T cells, is usually a result of the interaction of two coexpressed molecules on activated cells: Fas (CD 95) and FasL.

Not all forms of lymphocyte apoptosis are a consequence of activation. In fact, many lymphocytes are programmed to die unless protected by receptor-mediated stimulation or growth. This type of programmed cell death is due to neglect and does not appear to involve the Fas/TNF receptor family. The susceptibility of proliferating T cells for apoptosis positions IL-2 as a key, but generally unrecognized, regulator of T cell apoptosis (82). The fate of cycling T cells is thus linked to the prevailing state of the immune response. Without continuous antigen stimulation, the expression of IL-2 and its receptor falls, and passive or 'lymphokine withdrawal' apoptosis ensues.

Cell death due to passive lymphokine withdrawal may result from the cytoplasmic activation of caspases regulated partly by mitochondria and the Bcl-2 protein. Therefore, passive apoptosis decreases the expanded population of the T cells at the end of an immune response (83). Conversely, if cycling T cells are strongly stimulated by antigen, active or 'antigen-induced' apoptosis occurs, which can be due to Fas ligand and TNF- $\alpha$ . Even though cycling T cells are programmed to die after strong antigen re-engagement, effector functions, such as lymphokine production and T cell cytotoxicity, are still potently expressed (79). The net effect is a fine-tuned feedback response for eliminating T cells if there is too much or too little antigen and IL-2 (Fig. 5).

O'Sullivan and others have shown that impairment of the adaptive immune response after traumatic or thermal injury is characterized by failure of IL-2 production (84, 85), which is produced by T<sub>H1</sub> cells. Major injury leads to a predominance of T<sub>B2</sub> cells; because expression of IL-2 is decreased during major injury, the passive or 'lymphokine withdrawal' apoptosis of T cells ensues. This process may explain why TNF- $\alpha$  and FasL



**Figure 5.** Pathways leading to T cell apoptosis. T cells undergo apoptosis either actively by antigen stimulation, leading to expression of Fas, TNF- $\alpha$ , and IL-2, or passively by lymphokine withdrawal.

do not appear to be directly involved in injury-induced apoptosis. It is presumed that this increased apoptotic loss of T lymphocytes further increases the susceptibility to sepsis that is manifested in severely injured patients. Furthermore, it has been shown that increased lymphocyte apoptosis in peripheral blood T cells from burned patients appears to contribute to decreased lymphocyte immune responsiveness (86).

#### APOPTOSIS IN LYMPHOID ORGANS IN SEPSIS

A recent clinical study by Hotchkiss and colleagues demonstrated increased apoptosis in lymphocytes (spleen and lymph nodes) and gastrointestinal columnar epithelial cells (colon and ileum), as well as a pronounced lymphopenia in patients who died from sepsis (87). In addition, marked increases in activated caspase-3 and reduced Bcl-2 expression were also seen in these tissues. In contrast, patients dying from non-septic causes did not show an increase in apoptosis in any of these cell populations, nor was there a similar increase in caspase-3 activity (87). This organ-associated lymphoid cell apoptosis is consistent with clinical re-

ports indicating an increased frequency of lymphopenia in hospital patients with sepsis (88). The presence of lymphopenia has been documented to correlate with an adverse outcome (89).

Similar findings have been seen in murine models of sepsis (90). Rodents subjected to either a scald burn injury or generalized peritonitis secondary to a cecal ligation and puncture demonstrated a very rapid onset of increased apoptosis in lymphoid organs, usually within 3 h (spleen, thymus, and bone marrow) (Table 1). We reported that after a scald burn injury, caspase-3-dependent apoptosis increased in lymphoid organs, and up to 35% of T cells in the thymus were undergoing apoptosis (91). Similarly, Ayala and colleagues observed increased apoptosis in both mature and immature T cell populations from thymus, spleen, and bone marrow of mice after a cecal ligation and puncture (92). Hotchkiss also observed that the increased apoptosis in lymphoid organs appeared to play a direct role in the adverse outcome to a cecal ligation and puncture, since selective Bcl-2 overexpression in T lymphocytes or a systemic administration of caspase inhibitors led to a decrease in apoptosis in thymus and spleen, as well as increased survival (90, 93).

Although there is a consensus that apoptosis is increased in lymphoid cell populations during sepsis syndrome (94), the humoral or endocrine factors that stimulate this increased apoptosis are still not fully known. For example, Ayala and his group have reported in rodents that thymocyte apoptosis seen during polymicrobial sepsis is primarily a direct response to corticosteroids and can be controlled *in vivo* by the steroid receptor blocker mifepristone. Furthermore, thymocyte-induced apoptosis was Fas ligand and TNF- $\alpha$  independent (95, 96). We came to similar conclusions regarding the increased apoptosis in thymus and spleen after a scald burn injury, since treatment with mifepristone blocked the increased apoptosis in these tissues (97). The increased apoptosis was still seen in endotoxin-resistant mice (C3H/HeJ) and in mice with deletions or mutations in TNF- $\alpha$  ( $tnf^{-/-}$ ) and FasL ( $gld$ ).

The role played by TNF- $\alpha$  and FasL in sepsis-induced lymphoid cell apoptosis remains controversial and may depend on the sepsis model studied. TNF- $\alpha$  appears to play a role in thymocyte-induced apoptosis in animals

**Table 1.** Organ apoptosis in rodent models of sepsis<sup>a</sup>

	Animal model	Thymus	Spleen	Liver	Lung	Gut
Fukuzuka, 1999 (91)	Scald burn	++	++	-	+	-
Mignon, 1999 (102)	LPS/D-Gal			++		
Chung, 1998 (99)	Cecal ligation and puncture					++
Hotchkiss, 1997 (90)	Cecal ligation and puncture	++	++	±	+	+
Hiramatsu, 1997 (107)	Cecal ligation and puncture	++	+		+	+
Ayala, 1996 (92)	Cecal ligation and puncture	++				
Rogers, 1996 (124)	Listeria monocytogenes				++	
Wang, 1994 (125)	Gram-negative bacteria					
Ogasawara, 1993 (126)	anti-Fas antibody	++			++	

<sup>a</sup> ++, Extensive apoptosis; +, apoptosis present; ±, modest apoptosis; -, apoptosis absent.

injected with high-dose lipopolysaccharides (98). This may also be cell type specific, since a decrease in both intraepithelial gut lymphocyte apoptosis and in mortality was seen in FasL-deficient mice (*gld*), suggesting FasL to be an important contributor to survival and the loss of lymphoid tissues from the gut (99). An additional study also suggested FasL as a mediator for splenocyte apoptosis, contributing to the depression of splenocyte immune responses seen during polymicrobial sepsis (100).

### APOPTOSIS IN PARENCHYMAL CELLS

Although there is general agreement that apoptosis is increased in lymphoid cells during sepsis, data supporting a significant parenchymal cell apoptosis in solid organs during sepsis are much less convincing. In a murine endotoxemia model, endogenously produced TNF- $\alpha$  induced massive hepatocyte apoptosis and death; however, hepatocyte apoptosis required the simultaneous presence of transcriptional inhibition by either D-galactosamine or actinomycin-D (101). Caspase-3 activity was essential for TNF- $\alpha$ -induced hepatic parenchymal cell apoptosis, since pretreatment of mice with synthetic substrates for caspase-3 (DEVD-CHO) prevented mortality (102). This increased hepatocellular apoptosis was an important signal for the transmigration of primed neutrophils sequestered in sinusoids (101, 103). TNF- $\alpha$  induced neutrophil sequestration in hepatic sinusoids during sepsis and endotoxemia, which was also associated with hepatocyte necrosis (104).

Similarly, in a rodent model of endotoxemia after priming with *P. acnes*, blocking Fas ligand and TNF- $\alpha$  prevented hepatocyte apoptosis and death (105). Moreover, overexpression of hepatitis B antigen induced FasL-dependent hepatocyte apoptosis.

Despite the evidence that hepatocyte apoptosis is increased in some models of experimental endotoxemia, hepatocyte apoptosis appears not to be a common occurrence in other models of sepsis or endotoxemic shock. High-dose endotoxin-induced mortality is not associated with significant hepatocyte apoptosis, although hepatocyte necrosis is widespread (106). In an animal model of generalized peritonitis (cecal ligation and puncture), the presence of widespread hepatocellular apoptosis is controversial. Hiramatsu was unable to see any significant increase in hepatocyte apoptosis in mice after a cecal ligation and puncture (107). We have examined apoptosis and caspase-3 activities in the liver of mice after a cecal ligation and puncture, and have found only very modest increases when compared to endotoxin and transcriptional inhibition or to FasL activation (data not shown). We recently surveyed mouse solid organs for increased apoptosis 3–24 h after a scald burn injury and found no evidence of increased solid organ apoptosis or caspase-3 activity in liver, lungs, kidney, or skeletal muscle (91).

Hotchkiss and colleagues examined parenchymal cell apoptosis in patients who had immediately died

from sepsis and found increases in apoptosis that were limited to epithelial cells of the gut (87). Although apoptosis was markedly increased in gut lymphoid tissues of the spleen and thymus, increased apoptosis was not seen in parenchymal cells from either the heart, lungs, kidney, or liver.

### NEUTROPHIL APOPTOSIS DURING SEPSIS

Neutrophils are inflammatory cells with potent oxidative and proteolytic potential that are usually the first line of defense against invading pathogens. Activated neutrophils produce cytotoxic factors leading to deleterious inflammatory processes, including tissue injury (108).

While lymphoid cells are undergoing accelerated apoptosis, spontaneous neutrophil apoptosis associated with sepsis or SIRS is delayed (109, 110). This decreased apoptosis is thought to be important in enhancing tissue injury in ARDS, SIRS, and burn injury by promoting a disbalanced tissue load of neutrophils and uncontrolled release of toxic metabolites injurious to endothelial cells' mitochondria and collagen (109, 111). Bacterial products and cytokines released during sepsis can delay neutrophil apoptosis and delayed neutrophil apoptosis has been associated with severe clinical sepsis (110, 112). In trauma patients, neutrophil apoptosis correlated with MODS in these patients (113). Further evidence of decreased neutrophil apoptosis during sepsis was shown in a study where plasma of patients with SIRS decreased apoptosis in neutrophils from healthy individuals. Neutralization of GM-CSF and addition of IL-10 attenuated the delayed apoptosis (114). Delayed neutrophil apoptosis after exposure with GM-CSF appears to be in part due to phosphorylation of Bad, which results in the dissociation of Bad from other members of the Bcl-2 family, leading to prolonged cell survival. Anti-inflammatory cytokines such as IL-10 can reverse the anti-apoptotic effect of SIRS on neutrophil life span (110, 114), whereas proinflammatory cytokines like IL-1 and IL-6 prolong survival of neutrophils by inhibiting apoptosis (115). IL-10 has been reported to end pulmonary inflammation *in vivo* by promoting neutrophil apoptosis (116). Glucocorticoids inhibit apoptosis of human neutrophils, which might be another reason for the decreased apoptosis in these cells (117).

### THERAPEUTIC OPTIONS

Although anti-apoptotic therapies for septic patients are presently unavailable, further exploration of this clinical approach appears warranted. The pro- and anti-apoptotic pathways regulating T cell and B cell death are rapidly being delineated, not only in lymphoproliferative diseases, but also in acute inflammation (83). Therapeutic targets critical for inducing lymphocyte apoptosis include, among others, caspase-8, caspase-3, and PARP. Alternatively, stimulating the overexpression of anti-apoptotic proteins like Bcl-2,

Table 2. Different pro- and antiapoptotic factors involved in the cell death

Proapoptotic factors	Antiapoptotic factors
Bax	Bcl-2 (B cell lymphoma 2)
Bcl-x <sub>s</sub>	Bcl-x <sub>L</sub>
Bik	IAP (inhibitor of apoptosis proteins)
Bid	
Bik (related to human Bik)	
Bak	
AIF (apoptosis-inducing factor)	
Apaf-1 (apoptosis protease-activating factor 1)	
FasL (Fas ligand)	
TNF $\alpha$ (tumor necrosis factor $\alpha$ )	
Granzyme B	

Bcl-x<sub>L</sub> or IAP or inhibiting the expression of pro-apoptotic proteins like Bid and Bax could be appropriate therapeutic targets (Table 2 and Fig. 4).

Several formidable challenges must be overcome in order to develop anti-apoptotic therapies in the septic patient. These include successfully targeting both the appropriate signaling pathway and the specific cell population. There have been several attempts at blocking the circulating mediators or humoral factors during sepsis that induce apoptosis, like TNF- $\alpha$ , FasL, granzymes, or glucocorticoids. Although this is the most technically feasible approach, it has not proved successful in treating the increased lymphocyte apoptosis that characterizes sepsis syndromes. This is due partly to the observation that there is considerable redundancy in actions among these mediators. For example, TNF- $\alpha$ , FasL, and glucocorticoids are all pro-apoptotic, and their expression or release is increased acutely in sepsis syndromes (13, 96). In fact, we have shown that early after a scald burn injury or generalized peritonitis in the mouse, increased TNF- $\alpha$  and FasL expression has been documented in the thymus and spleen where increased T cell apoptosis is occurring. In these same experimental models of sepsis and burn injury, however, blocking TNF- $\alpha$  and FasL had only minimal effects on lymphoid apoptosis in spleen, thymus, and gut-associated lymphoid tissue. Inhibiting glucocorticoid receptor binding was more effective (96). Glucocorticoid blockade, unfortunately, is not a feasible approach for treating the septic patient because of its obligatory role in carbohydrate and energy homeostasis in stressful conditions.

A more appropriate schema is to target directly the specific intracellular pathways and effectors leading to sepsis-induced cell death, such as the caspases or PARP, whose activation may be the common product of mitochondrial or cytosolic apoptotic pathways. These more distal targets may provide greater specificity than the humoral mediators that induce them, since they repre-

sent common intracellular signaling pathways for diverse extracellular mediators.

One experimental approach to prevent apoptosis in animals has been to forcibly overexpress endogenous proteins that interfere with apoptotic processes in lymphoid tissues. Overexpression of the anti-apoptotic Bcl-2 protein in T and B lymphocytes actually improved survival after a cecal ligation and puncture, and prevented apoptosis in lymphoid organs (118). Moreover, transgenic mice overexpressing Bcl-2 in gut epithelial cells were resistant to ischemia-reperfusion injury in the gut (119). In Fas-mediated acute liver injury, apoptosis of hepatocytes can be decreased *in vivo* in different ways. Bcl-2-overexpressing hepatocytes of transgenic mice show decreased liver apoptosis (120). Also, Bid-deficient mice have a higher survival rate and a decreased hepatocellular liver injury after injection of ant-Fas antibody compared to wild-type mice (72). Deletion of either pro-apoptotic protein suggests a beneficial role. Furthermore, caspase inhibitors such as ZVAD have been shown to efficiently block Fas-mediated liver destruction (121). Alternative approaches include overexpression of the natural inhibitors of caspases, including the IAP family of proteins.

The second major challenge will be to target *only* the lymphocyte and transiently block the increased apoptosis *only* during sepsis. Since apoptosis is a normal physiological process essential for the death and removal of several cell populations of lymphocytes, delayed apoptosis has been implicated in the malignant transformation of several cell populations, including B lymphocytes (122). In addition, delayed apoptotic removal of neutrophils has been imputed in the pathogenesis of adult respiratory distress syndrome (111). Inhibition of neutrophil apoptosis through NF- $\kappa$ B-dependent pathways in sepsis or SIRS appears to prolong the life of neutrophils once they have extravasated the blood compartment into lung parenchymal tissues and to potentially increase oxidative damage in the lung (123). Thus, targeted blockade of apoptosis in lymphocyte populations must be specific enough to target primarily those lymphocyte cell populations undergoing increased apoptosis, and to be sufficiently transient to prevent the risk of malignant transformation associated with prolonged blockade of cell death.

## CONCLUSIONS

Apoptosis, or programmed cell death, is characterized by nuclear degeneration, condensation, and nuclear DNA degradation; phagocytosis of cell residua. MODS or even MOF are often associated with an increased rate of apoptosis in lymphoid cells and, to a lesser extent, in organ parenchyma. Therapeutic efforts aimed at blocking cell signaling pathways leading to apoptosis may represent a new therapeutic target for the critically ill patient with sepsis or systemic inflammatory response syndromes.

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